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Carduus nigrescens Seed Oil—A Rich Source of Pentacyclic Triterpenoids[1]

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ABSTRACT

Pentacyclic triterpene alcohols (3%), their acetates (18%), and their long chain fatty acid esters (11%), together with triterpene acids (18%), represent ca. 50% of the oil from the seed and pericarp of the thistle *Carduus nigrescens* Vill. (Compositae). Along with the usual fatty acids, alkaline hydrolysis of this oil gave triterpene alcohols, some of which were identified by gas chromatography-mass spectrometry. Composition of the triterpenoid fraction, as indicated by gas chromatography of the corresponding acetates, was: α-amyrin (6%), β-amyrin (15%), lupeol plus ψ-taraxasterol (3%), erythrodiol (6%), and oleanolic acid (3%). Several components, representing 16% of the oil, were not identified. The content of pentacyclic triterpenoids is the largest found in plant seed oils.

INTRODUCTION

Carduus nigrescens Vill. is a member of the family Compositae, tribe Cynareae. Genera of this tribe are commonly known as thistles. The seed and pericarp of *C. nigrescens* contain 41% oil. Preliminary analysis of this oil in our laboratory indicated the presence of large amounts of nonglyceride components not usually associated with seed oils (unpublished results). In addition to maxima usually associated with triglycerides, the IR spectrum showed a strong absorbance at 8.1 μm (1230 cm^{-1}) which is characteristic of acetates. Gas liquid chromatography (GLC) revealed constituents which had retention characteristics resembling those of triterpene alcohols, triterpene acetates, and long chain fatty acid esters of triterpenes, as well as the common triglycerides.

Only a few references appear in the literature (1-3) that deal with the analysis of seed oils of plants of the genus *Carduus*, but none of these discuss the nonglyceride components. Pentacyclic triterpenoids have been reported in various plant parts of the Cynareae (4-6), but in only one publication have they been reported in the seed oils. In 1967, Mikolajczak and Smith (6) found 40% pentacyclic triterpene alcohols in the seed oils of *Jurinea anatolica* and *J. consanguinea.*

This paper describes the composition of *C. nigrescens* seed oil and identifies its major triterpenoid constituents.

EXPERIMENTAL METHODS

Reference Materials

The following authentic compounds were used as reference materials in thin layer chromatography (TLC), GLC, and mass spectrometry: acetates of α-amyrin, β-amyrin, ψ-taraxasterol, lupeol, and methyl oleanolate. α-Amyrin acetate was prepared (6) from the free alcohol purchased from K&K Laboratories, Plainview, N.Y. β-Amyrin acetate was donated by P. de Mayo, University of Western Ontario. Acetates of ψ-taraxasterol and lupeol were presented by E.R.H. Jones, Oxford University. Acetyl methyl oleanolate was prepared from oleanolic acid given by R.M. Parkhurst, Stanford Research Institute, Menlo Park, Calif.

Extraction and Analysis of Oil

Seed extraction: C. nigrescens seeds (6.17 g, including pericarp) were ground and extracted 6 hr in a Soxhlet extractor with petroleum ether (bp 35-60 C). Solvent was removed under vacuum at 25-30 C to give a bright yellow viscous oil (2.53 g).

Methylation of free acids: Free acids in the original oil were converted to methyl esters by treatment with an ethereal solution of diazomethane (7).

GLC analysis: GLC of the original oil and the methylated oil was carried out with a Hewlett-Packard model 5750 gas chromatograph equipped with a 3 ft x 1/8 in. stainless steel column packed with 3% OV-1 on 100-120 mesh Gas Chrom Q (Applied Science Laboratories, State College, Pa.). The temperature was programed at 4 C/min 100-400 C. The injection port was operated at 300 C and the flame ionization detector at 360 C. Retention data are reported as relative retention time (RRT) with β-amyrin acetate as the reference compound (Fig. 1). Methyl esters were identified by their equivalent chain length (8). Quantities of all components are expressed as GLC area percentages.

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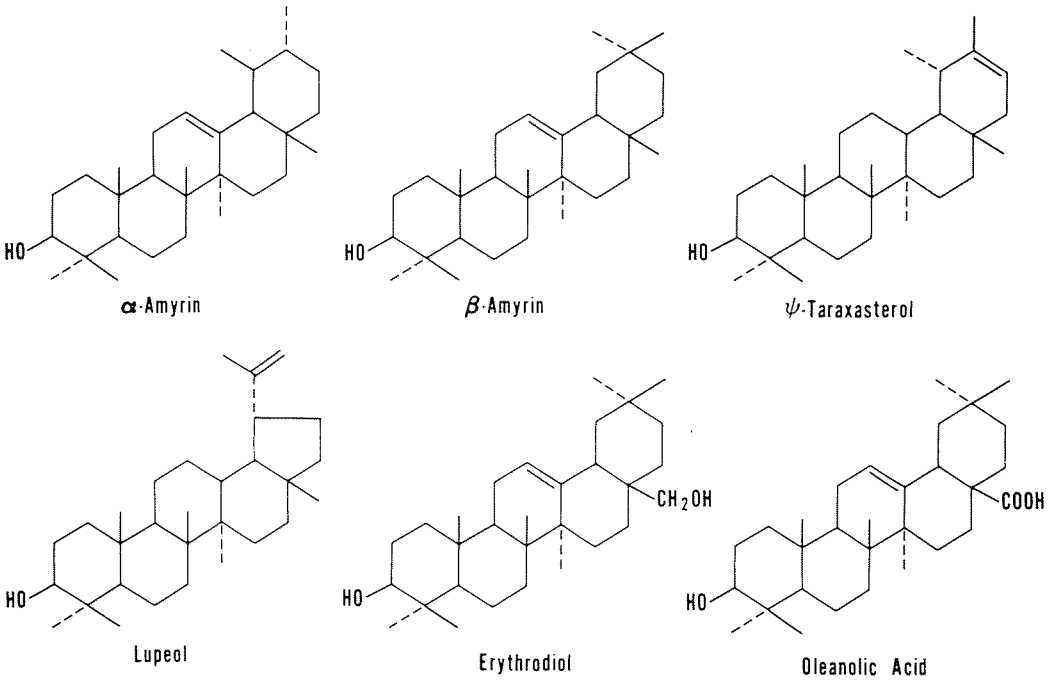

FIG. 1. Pentacyclic triterpenoids of *Carduus nigrescens* seed oil.

Preparation and Analysis of Mixed Methyl Esters

Preparation: Mixed methyl esters of the constituent acids in *C. nigrescens* seed oil were prepared by dissolving a 100 mg portion of the oil in 3 ml benzene and refluxing 4 hr with 5 ml 0.5N sodium hydroxide in methanol. Upon cooling, the alkaline solution was extracted with hexane to remove alcohols and other unsaponifiable material. Hexane extracts containing the alcohols were set aside for later examination. To the alkaline solution was added 5 ml boron trifluoride-methanol reagent of Metcalfe, et al. (9). After refluxing the mixture for 10 min, 50 ml aqueous saturated sodium chloride was added. The esters were extracted with hexane, and solvent was evaporated to give a sample of methyl esters.

GLC analysis: GLC of the mixed methyl esters was conducted on an F&M model 402 gas chromatograph as described by Kleiman, et al. (10).

Acetates of Triterpene Alcohols

Preparation: The hexane extracts set aside during the methyl ester preparation were evaporated to provide a mixture of alcohols, which was treated by the acetylation procedure described by Mikolajczak and Smith (6).

Preparative TLC: Preparative TLC of the mixed acetates was carried out on glass plates coated with 1 mm layers of Silica Gel G impregnated with 20% silver nitrate. The developing solvent was benzene-hexane (40:60). Sample bands were located by viewing the plates under long-wave UV light after they had been sprayed with a 0.2% solution of 2′,7′-dichlorofluorescein. Individual fractions were eluted with benzene-hexane (1:1). Fractions were numbered consecutively on the basis of increasing R_f.

Analytical TLC: Analytical TLC of the mixed acetates and of the fractions from preparative TLC was conducted with benzene-

TABLE I

Composition of Diazomethane-Treated *Carduus nigrescens* Seed Oil

Component	RRT[a]	GLC[b] area, %
Methyl esters of free fatty acids	0.10-0.67	19
Unknowns	0.70-0.80	1
Triterpene alcohols	0.90-0.95	3
Acetates of triterpene alcohols	1.00-1.02	18
Acetates of triterpenoid acid methyl esters	1.07-1.13	18
Diglycerides	1.18-1.27	4
Long chain fatty acid esters of triterpene alcohols	1.33-1.57	11
Triglycerides	1.62-1.86	26

[a]Relative retention time: The ratio of the retention time of an individual component to that of β-amyrin acetate.

[b]GLC = gas liquid chromatography.

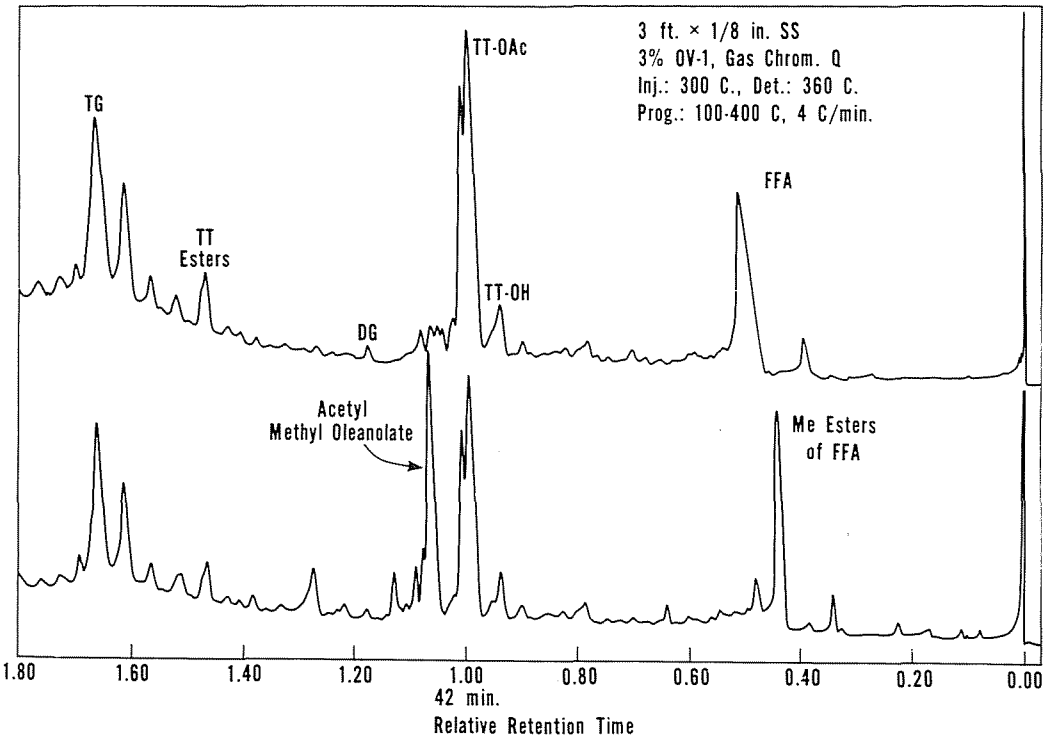

FIG. 2. Gas liquid chromatogram of *Carduus nigrescens* seed oil. Top: Original oil. Bottom: Oil after treatment with diazomethane. TG = triglycerides; TT esters = long chain fatty acid esters of triterpene alcohols; TT-OAc = triterpene acetates; FFA = free fatty acids; Me esters of FFA = methyl esters of free fatty acids.

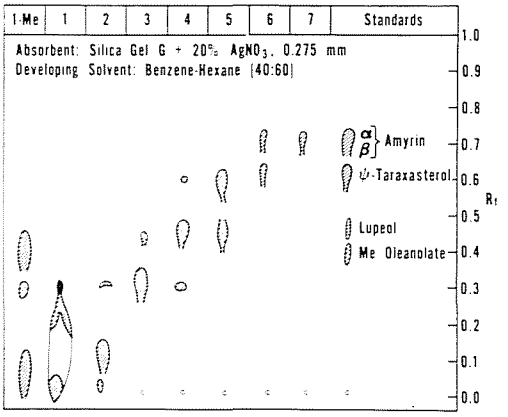

FIG. 3. Analytical thin layer chromatogram of triterpenoid acetate fractions (1 Me = fraction 1 after treatment with diazomethane).

hexane (40:60) on glass plates coated with 0.275 mm layers of Silica Gel G impregnated with 20% silver nitrate. The spots were visualized by charring with a sulfuric acid-dichromate solution. R_f values of individual components were compared with those of authentic reference compounds.

GLC analysis: The mixed acetates and preparative TLC fractions were examined by GLC with the Hewlett-Packard model 5750 instrument as described above. Fractions which appeared to contain free acids were treated with diazomethane and reexamined by GLC and TLC.

GLC-coupled mass spectrometry (GC–MS): Mass spectra of the acetylated fractions were obtained with a DuPont 21-492-1 mass spectrometer. Samples were introduced into the mass spectrometer through a Bendix 2600 gas chromatograph and a stainless steel jet separator. The gas chromatograph was equipped with a 3 ft x 1/8 in. stainless steel column packed with 3% Dexsil 300 on Gas Chrom Q. The temperature was programed at 4 C/min 230-350 C. The transfer line and jet separator were held at 280 C, and the mass spectrometer source was kept at 260 C. The filament current was 250 μA and the ionizing voltage, 70 eV.

Total Methyl Ester and Acetate Mixture

A 100 mg sample of the oil in 3 ml benzene was refluxed 3 hr with 10 ml 5% anhydrous HCl-methanol. The product, isolated by conventional methods, contained methyl esters and

TABLE II

Mass Spectra of Acetates of Triterpenoid Alcohols from *Carduus nigrescens* Seed Oil

Alcohol	Mass spectrum, *m/e* (relative intensity)
α-Amyrin	189(16), 203(11), 218(100), 249(1.4), 408(2.7), 453(1.2), 468(M^+)(4.4)
β-Amyrin	189(12), 203(28), 218(100), 249(1.3), 408(1.6), 459(0.59), 468(M^+)(2.4)
Lupeol	189(100), 203(28), 204(27), 218(21), 229(11), 249(10), 365(7.8), 393(6.3), 408(11), 453(3.2), 468(M^+)(20)
ψ-Taraxasterol	189(100), 203(12), 204(5.6), 218(5.0), 229(7.3), 249(10), 365(4.9), 393(4.6), 408(17), 453(1.9), 468(M^+)(13)
Erythrodiol	189(24), 203(100), 216(63), 276(9.3), 406(6.2), 466(11.9), 526(M^+)(0.8)
Unknown C	55(59), 69(87), 81(100), 93(60), 119(60), 135(55), 145(31), 161(24), 175(26), 189(83), 203(38), 215(15), 276(4.6), 406(4.6), 423(13), 466(49), 526(M^+)(2.3)
Methyl oleanolate	189(34), 203(100), 249(12), 262(71), 437(2.8), 452(8.5), 512(M^+)(2.8)

triterpene alcohols; it was treated by the acetylation procedure used previously (6) and was analyzed by GLC.

IR Analysis

IR spectra were determined with Perkin-Elmer Infracord model 137 and model 377 instruments. The oil was analyzed as a thin film on NaCl disks. All other samples were run as 1% CCl_4 solutions in 1 mm NaCl cells.

RESULTS

Composition of Oil After Methylation

GLC of the oil after methylation (Table I) shows, in addition to triglycerides, a variety of components ranging from long chain fatty acid esters of triterpene alcohols to methyl esters of fatty acids. The major peak among the long chain triterpene esters had the same RRT (1.50) as α- or β-amyrin palmitate. In addition, a peak was found whose retention time corresponded to that of an authentic sample of acetyl methyl oleanolate (Fig. 2).

Mixed Methyl Esters from Saponification

Saponification of the oil, followed by removal of unsaponifiables and subsequent treatment of the alkaline portion with boron trifluoride-methanol, gave methyl esters of common acids whose GLC analysis showed (amounts in parentheses are area percent): 12:0(1), 14:0(2), 16:0(12), 18:0(4), 18:1(26), 18:2(43), 18:3(5), 20:0(2), 20:1(2), 22:0(0.4), and 22:1(3).

Triterpene Alcohols

GLC analysis of the acetylated triterpene alcohol fraction indicated the presence of a complex mixture of triterpenoids. GC-MS of this mixture yielded spectra for the major components of the fraction; however, some minor components did not give sufficiently intense spectra for meaningful interpretation. Therefore, the acetate mixture was fractionated by preparative TLC. Seven fractions were separated with wt percentages as follows: 1(59), 2(5.4), 3(6.5), 4(7.6), 5(3.3), 6(4.2), and 7(13.6). There was considerable overlap in composition among these TLC bands (Fig. 3), but GC-MS of these partially purified materials provided usable spectra for minor triterpenoid components. Fractions 6 and 7 each were composed primarily of α- and β-amyrin acetates which were identified by TLC, GLC, and MS (Table II). In contrast, the acetates of ψ-taraxasterol in fractions 5 and 6 and lupeol in fractions 4 and 5 gave identical GLC retention times and similar mass spectra. However, they were readily distinguishable by analytical TLC. The similarities in the mass spectra and the GLC retention times were also observed with authentic samples of lupeol and Ψ-taraxasterol acetates. Their spectra resembled the mass spectrum obtained by Budzikiewicz, et al., (11) for ψ-taraxasterol acetate. Lupeol can be isomerized to ψ-taraxasterol, and certain transformation products can be derived from both of these compounds through acid-catalyzed solvolysis reactions (12). Possibly, related interconversions occur during the rigors of GLC and MS and are responsible for the observed similarities.

The major component of TLC fraction 3 had a mass spectrum (Fig. 4) closely resembling that published for erythrodiol diacetate (11). The major component of fraction 2 (unknown C, Table III) gave a mass spectrum (Fig. 4) in which the fragmentation pattern and molecular ion were the same as those observed for erythrodiol diacetate, but the relative intensities for the ions of *m/e* 189 and 203 were different. The peak at *m/e* 276 might be furnished by retro Diels-Alder fragmentation of ring C (11). These observations suggested that unknown C is an isomer of erythrodiol diacetate with a 12,13-double bond. To our knowledge, only one other mass spectrum has been published for

TABLE III

Composition of *Carduus nigrescens* Seed Oil after Methanolysis and Acetylation

Component	RRT[a]	R_f of acetate	GLC[b] area, %
Methyl esters of fatty acids	0.15-0.67		50
Unknowns	0.68-0.90		10
Unknown A	0.96		1
β-Amyrin acetate	1.00	0.70	15
α-Amyrin acetate	1.01	0.70	6
ψ-Taraxasterol acetate	1.04	0.60	3
Lupeol acetate	1.04	0.45	
Acetyl methyl oleanolate	1.06	0.40	3
Erythrodiol djacetate	1.10	0.30	6
Unknown B	1.12		2
Unknown C	1.14	0.10	1
Unknown D	1.17		2
Unknown E	1.18		1

[a]Relative retention time: The ratio of the retention time of an individual component to that of β-amyrin acetate.

[b]GLC = gas liquid chromatography.

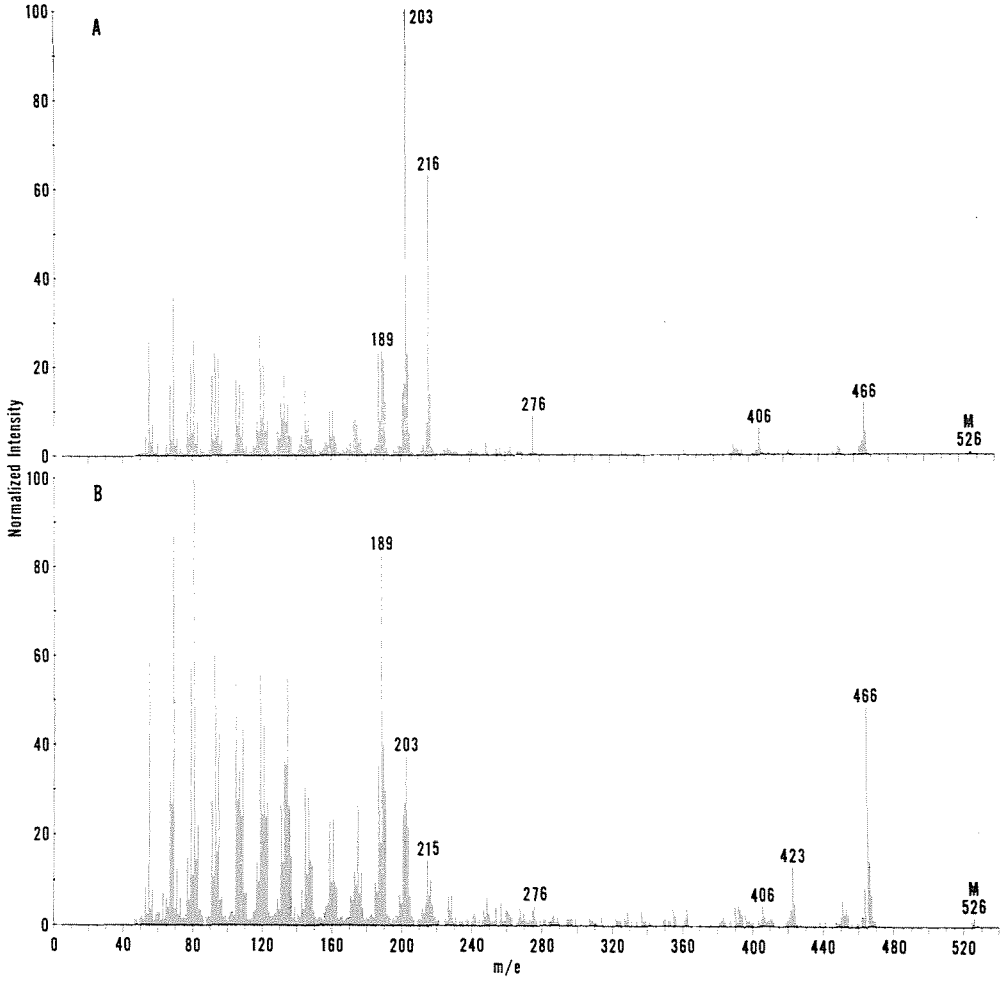

FIG. 4. Mass spectra of acetates of: (A) erythrodiol and (B) unknown C from *Carduus nigrescens* seed oil.

an isomeric Δ^{12}-ursene or oleanenediol diacetate–that of 30-hydroxy-β-amyrin diacetate (11). However, the relative intensities of its peaks do not match those in the spectrum of unknown C.

IR and GLC showed that free acids were present in the mixed acetates and were concentrated in TLC fraction 1; evidently, this acidic material was carried along during extraction of "unsaponifiables" with hexane. After methylation of the mixed acetates and fraction 1, analytical TLC revealed a spot at R_f 0.40 that was not originally observed. GLC showed a new component in the esterified mixed acetates, RRT 1.06, which constituted the major portion of the esterified fraction 1. The mass spectrum of this material closely resembled that of a known sample of acetyl methyl oleanolate. The mass spectrum of the corresponding acetyl derivative of methyl ursolate, an isomeric triterpene, did not match the spectrum of any component in this mixture.

Hydrocarbons and Sterols

Hydrocarbons may have been present in small amounts in the unsaponifiable fraction of the oil, but they were not investigated. Neither were sterols found in many plant seed oils (13-16) identified.

DISCUSSION

Pentacyclic triterpenoids have been found as minor components in many common plant seed oils (13-16) and often represent the greater part of the unsaponifiable portion of these oils. They were usually associated with hydrocarbons, sterols, and other terpene alcohols, especially with cycloartenol and 24-methylenecycloartanol.

Jacini, et al., (13) as well as Fedeli and Jacini (14) reported the composition of the nonglyceride components of 18 common vegetable oils and found 2 that contained α-amyrin and 10 with β-amyrin. Itoh, et al., in 1973 (15) studied the unsaponfiables of 19 common vegetable oils and reported α-amyrin in 6 and β-amyrin in 17 of these. In 1974, the same group (16) reported α-amyrin, β-amyrin, and lupeol in eight other seed oils. In all these cases, the pentacyclic triterpenes were minor constituents (less than 1% of the oil). In 1961, Vioque and Morris (17) reported that oleanolic acid occurs in olive oil. Our results show that this triterpene acid occurs in *C. nigrescens* seed oil as an acetate, but with the carboxyl groups free.

At the 50% level, *C. nigrenscens* seed oil has the highest concentration of pentacyclic triterpenoids of any known seed lipid. The highest reported previously was 40% in *J. anatolica* and *J. consanguinea* (6). *C. nigrescens* seed oil is also one of the rare examples of a seed oil in which nonglyceride components predominate.

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